Low Molecular Weight Heparin and Unfractionated Heparin Are Both Effective at Accelerating Pulmonary Vascular Maturation in Neonatal Rabbits

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Background—Creation of a bi-directional cavopulmonary shunt after the Norwood procedure for hypoplastic left heart syndrome is delayed to allow pulmonary vascular resistance to fall with maturation of the pulmonary vascular bed. We hypothesized that unfractionated heparin (UFH) and low molecular weight heparin (LMWH), which promote angiogenesis and inhibit smooth muscle cell growth, could accelerate this process.

Methods and Results—Fifty-six newborn rabbits were randomly selected to receive UFH 225U/kg (n=12), LMWH 1 mg/kg (n=14), LMWH 10 mg/kg (n=16), or saline (n=14) by subcutaneous injection every 12 hours for 14 days. Treatment with heparin reduced mean pulmonary artery (PA) pressure by 12% to 16% relative to controls [9.0±0.2 (UFH), 9.4±0.1 (LMWH 1 mg/kg), 9.2±0.2 (LMWH 10 mg/kg) versus 10.7±0.2 mm Hg (saline), P=0.0001]. Lower PA pressures were associated with reduced alveolar:arterial ratio consistent with enhanced pulmonary angiogenesis in heparin treated animals [8±1 (UFH), 13±2 (LMWH 1 mg/kg), 12±2 (LMWH 10 mg/kg) versus 23±5 (saline), P<0.03]. Reduced PA medial wall thickness and muscularization, two additional features of pulmonary vascular maturation, were also more evident in heparin treated animals. Mean PA pressures in 14-day-old rabbits treated with heparin were lower than those measured in control rabbits less than 7 weeks of age suggesting that heparin shortens the pulmonary vascular maturation process by over 60%.

Conclusions—These results indicate that both UFH and LMWH are effective at accelerating pulmonary vascular maturation in newborn rabbits. This raises the possibility that administration of heparin to children after the Norwood procedure might allow for earlier conversion to a bi-directional cavopulmonary shunt. (Circulation. 2003;108[suppl II]:II-161-II-166.)

Key Words: pulmonary artery ■ angiogenesis ■ anticoagulants ■ growth factors ■ heart defects ■ congenital
Unfractionated heparin (UFH) has been shown to promote angiogenesis in the context of coronary ischemia and cardiac development, and inhibits vascular SMC proliferation. Furthermore, UFH has been shown to reduce medial thickness in experimental hypoxia induced pulmonary hypertension. Low molecular weight heparin (LMWH) is a purified form of UFH with a longer half-life, improved bioavailability, and effective antithrombotic properties when given by intermittent subcutaneous injection. LMWH appears to retain the SMC inhibitory properties of UFH, however, differential effects on angiogenesis have been described.

We now show that LMWH and UFH are both effective at accelerating the normal post-natal fall in PA pressure in rabbits. This is associated with an increase in PA density, reduced muscularization of small PAs, and thinning of the medial layer of muscular PAs. Fibroblast growth factor-2 (FGF-2), a potential mediator of heparin’s effect on angiogenesis and SMC proliferation, is expressed in the lung tissue of newborn rabbits but levels are not altered by heparin treatment.

This study is the first to show than LMWH and UFH both accelerate pulmonary vascular maturation thereby suggesting a potential therapeutic strategy to permit earlier conversion to a BCPS after the Norwood procedure.

Methods

Animal Model

The Animal Care Committee at the Hospital for Sick Children approved the experimental protocol and all animals were treated according institutional guidelines. Newborn New Zealand white rabbits (n=56) from 6 different litters were randomized within 24 hours of birth to receive either UFH (porcine intestinal mucosa derived, Organon Teknika Inc) 225 U/kg (n=12), LMWH (Enoxaparin sodium, Aventis Pharmaceuticals) 1 mg/kg (n=14), LMWH 10 mg/kg (n=16), or an equivalent volume of normal saline (n=14) by subcutaneous injection every 12 hours for 14 days. At the end of the experimental period the animals were anesthetized with Ketamine (11 mg/kg, sc), inhaled oxygen, Isoflurane, and nitrous oxide titrated to prevent any response to painful stimuli while maintaining spontaneous ventilation. Lidocaine 1% solution was infiltrated in the right supra-clavicular area and a silastic catheter introduced into the pulmonary artery through the right internal jugular vein. Pulmonary artery pressure was measured, the animals were sacrificed by exsanguination under anesthesia and tissues prepared for analysis. Pulmonary artery pressures were also measured in untreated control animals (3 to 4 in each group) at 2, 3, 4, 5, 6, 7, 8, and 9 weeks of age to establish a normal time course for the postnatal fall in PA pressure.

Tissue Harvest and Preparation

The lungs were irrigated with heparinized saline delivered through the pulmonary artery until the effluent from the left atrium was clear. The right lung was flash frozen in liquid nitrogen and the left lung prepared for morphometric analysis by perfusion of the pulmonary artery with a pressurized barium-gelatin suspension and fixation with 10% formalin delivered through the trachea according to a previously described technique. This technique allows accurate evaluation of uniformly distended arterial vessels distinguished from venous structures by opacification with the barium-gelatin suspension. The left lung was embedded in paraffin and sections stained using the Movat technique. The right ventricle to left ventricle (RV/LV) mass ratio was determined by dissecting the right ventricular free wall from the left ventricle and septum.

Morphometric Analysis

To determine the effect of heparin treatment on PA structure, Movat stained lung sections were analyzed by observers blinded to the treatment group. The alveolar:arterial ratio was determined by counting the number of alveoli and barium-filled arteries from low power photomicrographs taken of 10 random fields from each lung section. Muscularization of peripheral pulmonary arteries was evaluated by classifying every barium filled vessel with an external diameter of 15 to 50 µm from each lung section as either nonmuscular, partially muscular, or muscular based on the presence of a smooth muscle cell layer between the internal and external elastic lamina. The number of muscular and partially muscularized arteries was expressed as a percentage of the total number of arteries. Medial wall thickness was assessed by measuring the thickness of the medial layer of every barium filled vessel with an external diameter of 50 to 200 µm from each lung section. The medial wall thickness from opposite sides of each vessel were added together and expressed as a percentage of the total external diameter.

Western Blot Analysis for FGF-2

Frozen tissue samples randomly selected from animals treated with saline (n=4) and UFH (n=4) were crushed in liquid nitrogen, homogenized in lysis buffer and centrifuged. The supernatants were collected, and samples of equal protein concentration were loaded onto Tris-Glycine gels for electrophoresis. Proteins were transferred to PVDF membrane, blocked with 5% nonfat milk, incubated with primary antibody (AF233NA, 0.2 µg/mL, R&D Systems), and then with secondary antibody (SC-2768, 1:10000 dilution, Santa Cruz Biotechnology). A second blot was treated with secondary antibody alone to ensure that the identified band represented specific binding by the primary antibody. A chemi-luminescence system (ECL Plus, Amersharm International) was used to generate autoradiographs of the membranes and densitometry was performed to quantify the signal obtained. Equal protein loading was confirmed by Coomassie blue staining of gels and Ponceau staining of membranes.

Statistical Analysis

All values are expressed as the mean±SEM. Statistical significance was established using the Chi square test when comparing proportions. A one-way analysis of variance (ANOVA) was used when comparing continuous variables with post-hoc testing by Fisher’s protected least significant difference method to evaluate differences between each group.

Results

Survival and Body Mass

Overall survival at the end of the experimental period was 59% (33 of 56) for the newborn rabbits randomized into each of the 4 groups. The cause of death in the vast majority of cases appeared to be neglect by the mother. Survival was not different (P=0.39) in animals treated with saline (8 of 14), UFH (9 of 12), LMWH 1 mg/kg (9 of 14), or LMWH 10 mg/kg (7 of 16). Two animals in the UFH group survived to the end of the experimental period but died from right atrial perforation before a PA pressure could be obtained. Two animals from the saline group and 1 from the LMWH 10 mg/kg group had inadequate tissue perfusion precluding morphometric analysis.

We looked for evidence that heparin treatment affected somatic growth. There was a trend toward higher body mass in the three heparin treated groups (UFH=251±24 g, LMWH 1 mg/kg=262±22 g, LMWH 10 mg/kg=257±21 g) when compared with saline treated controls (208±21 g) but this difference did not reach statistical significance (P=0.33).
Effect of Heparin on PA Pressure, and RV/LV Mass Ratio

If heparin treatment does accelerate maturation of the neonatal pulmonary vascular bed resulting in an important fall in PVR, this should be evident as a reduction in PA pressure. Therefore, we measured PA pressure in each group at the end of the 14-day treatment period. Fourteen days of treatment with UFH reduced mean PA pressure by 16% compared with those animals treated with saline (Figure 1). Similar reductions in mean PA pressure were seen in rabbits treated with either dose of LMWH.

The RV/LV mass ratio should parallel a reduction in PA pressure as RV hypertrophy regress. However, changes in RV mass lag behind a fall in PA pressure therefore differences may not be as apparent at any given time. In this experiment we found a trend toward reduced RV/LV mass ratio in the heparin treated groups (UFH 0.286±0.001, LMWH 1 mg/kg 0.288±0.001, LMWH 10 mg/kg 0.279±0.001) compared with saline controls (0.300±0.001) but the difference did not reach statistical significance.

Effect of Heparin on the Structure of the Pulmonary Vascular Bed

To determine if the reduction in PA pressure in heparin treated animals is associated with structural changes in the pulmonary vascular bed, morphometric analyses were performed on lung tissue sections from the rabbits randomized to the four treatment groups. The alveolar:arterial ratio was reduced by more than 60% after 2 weeks of treatment with UFH when compared with saline treated controls (Figure 2 A, 2B and Figure 3) indicating that arterial density increased relative to the number of alveoli. Similar reductions in the alveolar:arterial ratio were found in those animals treated with either dose of LMWH (Figure 3). Muscularization of small PAs (15 to 50 μm external diameter) was reduced by >40% in rabbits treated with UFH when compared with saline treated controls (Figure 4). Similar reductions were also present in the animals treated with either dose of LMWH. Medial wall thickness was reduced by >30% in rabbits treated with UFH or LMWH when compared with saline treated controls (Fig. 2C, 2D, and Figure 5).

Normal Time Course for Postnatal Fall in Pulmonary Artery Pressure

To evaluate how much 2 weeks of heparin treatment shortens the normal postnatal pulmonary vascular maturation process, untreated control animals were studied at weekly intervals from 2 to 9 weeks of age (Figure 6). Mean PA pressures equivalent to those observed after 2 weeks of heparin therapy were not seen in control animals until they reached 7 weeks of age indicating that the maturation process was shortened by approximately 67%.

Figure 1. Heparin reduces PA pressure in newborn rabbits. Mean PA pressures measured in newborn rabbits after 2 weeks of treatment with UFH 225 U/kg (UFH, n=7), LMWH 1 mg/kg (LMWH-1, n=9), or LMWH 10 mg/kg (LMWH-10, n=7), are lower than in saline treated controls (n=8, P=0.0001). There were no statistical differences between any of the heparin treatment groups. Bars represent mean±SEM.

Figure 2. Representative photomicrographs demonstrate increased arterial density (arrows) in a lung section from a newborn rabbit treated with UFH for two weeks (B) compared with a control animal treated with saline (A). Medial thickness of muscular arteries is reduced with UFH treatment (D) when compared with saline treated controls (C). Scale bars=50 μm.

Figure 3. Heparin increases arterial density in newborn rabbit lungs. The alveolar:arterial ratio, which is inversely related to arterial density, is lower in rabbits treated with UFH 225 U/kg (UFH, n=7), LMWH 1 mg/kg (LMWH-1, n=9), and LMWH 10 mg/kg (LMWH-10, n=6) relative to saline treated controls (n=6, P<0.03). There are no statistical differences between any of the heparin treatment groups. Bars represent mean±SEM.
Effect of Heparin on FGF-2 Expression

We suspected that FGF-2, a heparin binding angiogenic factor and SMC mitogen, might play a role in the observed effect of heparin on neonatal lung maturation. To study this hypothesis, FGF-2 protein levels in lung tissue extracts from the saline and UFH groups were evaluated using a Western blot analysis. We identified FGF-2 in the tissue extracts, however, the relative amount did not appear to be affected by heparin treatment (Figure 7).

Discussion

The results of this experiment demonstrate that the length of time required for the normal post-natal fall in pulmonary artery pressure can be significantly shortened in newborn rabbits by treatment with either UFH or LMWH. The most prominent structural change in the pulmonary vascular bed of newborn rabbits treated with heparin was a major reduction in the alveolar:arterial ratio consistent with an increase in arterial density. New vessel growth leading to a gradual decline in the alveolar:arterial ratio is a recognized feature of normal pulmonary vascular maturation but the mechanism is not understood. Vascular endothelial growth factor (VEGF) expressed by developing airways has been implicated as a mediator of neovascularization during lung embryogenesis. However, an important role for VEGF in postnatal pulmonary vascular development has not been shown. We were unable to demonstrate expression of this pro-angiogenic factor in lung tissue extracts from 2-week-old rabbits (data not shown).

The angiogenic potential of heparin has been previously reported. Heparin induces expression of the potent...
angioitic growth factor FGF-2 by smooth muscle cells.\textsuperscript{15} In addition to increasing the level of FGF-2 expression, heparin can also enhance the angiogenic activity of FGF-2. Heparin releases FGF-2 from the extra-cellular matrix were it appears to be stored in an inactive form.\textsuperscript{16} Heparin also appears to act as a co-factor in the binding of FGF-2 to its receptor\textsuperscript{17} and increases FGF-2 stimulated angiogenesis in a dose dependent manner.\textsuperscript{18} FGF-2 was present in lung tissue extracts from our experimental animals, but the levels did not appear to be altered by heparin treatment. Higher arterial density in the lungs from neonatal rabbits treated with heparin cannot be attributed to an increase in FGF-2 expression but the possibility remains that heparin enhances FGF-2 activity.

Differential effects of UFH and LMWH on angiogenesis have been described in certain experimental models.\textsuperscript{10,19} Under some circumstances, LMWH appears to be a relatively potent angiogenesis inhibitor.\textsuperscript{20} In the present experiment, the angiogenic effect of LMWH tended to be slightly less than with UFH, but the difference was not statistically significant. It is possible that some of the angiogenic components of UFH are not present in the low molecular weight fraction, but their overall importance in terms of lung maturation appears to be minimal.

The other morphological features of pulmonary vascular maturation include regression of muscularization of small arteries and thinning of the medial layer of muscular arteries. These processes are both accelerated by heparin in neonatal rabbits. The relative decrease in the amount of smooth muscle in the maturing pulmonary arterial tree is probably related to a SMC proliferation rate that does not keep pace with the growth rate of the pulmonary vasculature. Heparin may enhance this effect by directly inhibiting SMC proliferation. The anti-proliferative influence of heparin on SMCs has been studied extensively in the context of intimal proliferation after systemic arterial injury.\textsuperscript{7} However, heparin has also been shown to both prevent\textsuperscript{21} and reverse medial thickening of the PA associated with hypoxia induced pulmonary hypertension.\textsuperscript{9}

Many potential mechanisms for the inhibitory effect of heparin on SMC proliferation have been proposed. These include altered growth factor expression, altered extracellular matrix composition and matrix-degrading protease activity, altered transcription factor binding, and a direct effect on cell cycle progression.\textsuperscript{22} However, the influence of heparin on medial thickness and muscularization in neonatal rabbit lungs may be an indirect effect secondary to a reduction in PA pressure resulting from the increased arterial density. We have previously shown, in an experimental model of pulmonary hypertension, that simply reducing PA pressure can induce regression of medial thickening and muscularization.\textsuperscript{23}

The objective of our investigations is to work toward allowing a BCPS to be created safely at an earlier age. Based on our experiments, heparin appears to shorten the time required for the normal postnatal falls in PA pressure by over 60%. Clinical experience indicates that a BCPS can be safely performed in children over 4 months of age. This would suggest that heparin treatment could potentially allow the cut-off age for a BCPS to be lowered toward 6 weeks.

There are several limitations that should be considered when interpreting the results of this study. We used PA pressure as a substitute end point for PVR because of technical issues related to making accurate hemodynamic measurements in this small animal model. PVR would be a more sensitive endpoint that is independent of cardiac output and more directly related to the risk of a BCPS. Confirmation of these results in a larger animal model would allow more detailed hemodynamic data to be examined. A second important limitation is that the animals studied in this experiment had normal pulmonary vascular beds. Children with congenital heart disease may have abnormal pulmonary vasculature related to the in utero blood flow pattern.\textsuperscript{24} Pulmonary vascular maturation and the impact of heparin on this process in an abnormal pulmonary vasculature have not been investigated. Finally, clinical studies have failed to show a positive effect of heparin on neointimal SMC proliferation after balloon angioplasty despite numerous animal models showing promising results. There appears to be heparin independent pathways for smooth muscle cell proliferation in humans that do not exist in lower species.\textsuperscript{25} These pathways may also be relevant in the context of pulmonary vascular maturation.

In conclusion, our results demonstrate that UFH and LMWH both accelerate maturation of the pulmonary vascular bed in neonatal rabbits. If a similar effect on the pulmonary vasculature of newborn children with certain congenital heart defects can be achieved, an important reduction in the age at which a BCPS can be safely performed may be possible.

References
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